Moll. Res. 18: 169–181 (1997).

Optimum conditions using manganese as a shell marker for abalone age validation studies

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Keywords: manganese; time-marker; abalone; shell; cathodoluminescence; age validation

Abstract

Blacklip abalone, *Haliotis rubra* (L.), were immersed for 48 to 144 hours in buckets containing sea water to which $MnCl_2.4H_2O$ was added at concentrations ranging from 9.5 to 1890 mg L^{-1}, in order to determine the optimum concentrations for marking. Manganese concentrations above 945 mg L^{-1} over the immersion period were lethal to abalone. Cathodoluminescence microscopy was used to detect manganese mineralised within shell layers *in situ*. Although the average length and width of manganese carbonate marks at the outer growing margin of the abalone shell increased with higher concentrations, marks in the shell area under the spire appeared to be maximal at intermediate concentrations. Better marks were found at the growing edge than under the spire, although this appeared to depend on the seasonal timing of marking. Manganese concentrations of 378 mg L^{-1} or less in sea water for 48 h were not lethal to abalone after 10 days, but the scores of marks indicated a possible sub-lethal toxic effect of 144 h immersion at 272 mg L^{-1}. In a field trial we obtained 93% survival after 14 days of abalone exposed to 200 mg L^{-1} of manganese for 48 h and of these 60% showed marks in the spire region, where age is estimated, so this technique can be used in age validation studies. However, the effectiveness of manganese as a time-marker for increment analysis appears to depend on the growth of abalone when exposed to the marker and the ambient levels of manganese in the natural habitat.

Introduction

Layers formed in the calcified tissue of aquatic animals, such as otoliths of fishes and shells of gastropods, are often used to age individuals but, in each case, the temporal nature of growth increments requires validation (Beamish and McFarlane 1983). This is important for all species that are commercially harvested, as accurate age estimation allows for assessment of populations using age specific models, such as egg-per-recruit and yield-per-recruit (Day and Fleming 1992: Nash *et al.* 1994). Darker layers under the spire of abalone were suggested to be annual by Muñoz-Lopez (1976), and have been used to estimate the age in a number of species (Prince *et al.* 1988; Nash *et al.* 1994; Shepherd *et al.* 1995a; b), but without proper validation (Day and Fleming 1992). In Australia, the management of abalone stocks will remain somewhat subjective until the age structure of populations can be determined with confidence, as this will allow better estimates of population parameters such as mortality, and facilitate the use of age structured models.

Cross-sections cut through abalone shell expose a calcified outer prismatic and inner nacre layer (Nakahara *et al.* 1982; Mutvei *et al.* 1985; Dauphin *et al.* 1989). The nacre layer consists of stacks of brick-like tablets of aragonite with thin protein-sheets between the laminae. These stacks of laminae are interspersed with thick dark protein rich layers (Erasmus *et al.* 1994; Shepherd *et al.*

1995b). In the spire region, under the apex, the protein layers form "rings" (similar to rings in a tree) when the spire is ground down, and appear to be deposited regularly, between one and four times per year in different species (Muñoz-Lopez 1976; Prince *et al.* 1988; Nash *et al.* 1994; Shepherd *et al.* 1995a; b). Similar protein layers are laid obliquely near the growing margin (the edge of the body whorl) and correspond to growth checks in many species (Day and Fleming 1992). These growth checks consist of a thick layer of conchiolin protein material and either aragonite or calcite (Zaremba *et al.* 1996; Hawkes *et al.* 1996). They have been shown to be formed annually in *H. discus discus* from Japan (Sakai 1960; Kojima 1995), in *H. mariae* from Oman (Shepherd *et al.* 1995a) and perhaps also in other abalone species (Forster 1967; Poore 1972). However, they appear to be deposited irregularly in other species, and may be induced by seasonal, reproductive and/or disturbance events (Sakai 1960; Day and Fleming 1992). The timing and frequency of deposition of these growth checks within the nacreous layer of both the spire and edge regions require validation (Day *et al.* 1995).

To date, validation of the temporal nature of growth increments under the spire is based on the rings found in size classes of juvenile abalone assumed to be annual cohorts, or simply by comparing the graph of size versus number of rings to predicted growth curves (Prince *et al.* 1988; Shepherd *et al.* 1995a; b). This method cannot validate age classes beyond maturity because as growth slows, size no longer provides any information about age. Without proper validation of the periodicity of growth layers, the age structure of populations cannot be estimated with any confidence (Day and Fleming 1992; McShane and Smith 1992; Erasmus *et al.* 1994; Day *et al.* 1995).

Staining of calcified tissue with chemical tags, such as fluorochromes and strontium, has been used to validate the timing of growth layer deposition in order to determine ages of molluscs, sharks and fishes (Yamada 1971; Behrens and Mulligan 1982; McFarlane and Beamish 1987; Villiers and Sire 1985; Officer 1995). Pirker and Schiel (1993) and Day *et al.* (1995) have tested various fluorochromes to mark abalone shells, but although these stained the growing edge of abalone shells, they failed to stain the nacre under the spire where age is estimated (Day *et al.* 1995).

Behrens and Mulligan (1990) outlined the criteria for a chemical to be an effective tag. The stain must be non-lethal, rare in the animal and ambient medium, incorporated as a permanent mark in the body and detectable a year or more after marking. Behrens and Mulligan (1990) first suggested manganese may be useful as a chemical tag. They showed that manganese, introduced through artificial feed, could be detected in powdered bone samples of hatchery salmon, using X-ray fluorescence and microprobe analysis. However, the ability to detect a chemical tag *in situ* would increase its use as an effective chemical marker in increment studies. When a stain can be viewed within the calcified tissue it acts as a time-mark, so that the frequency of growth layers deposited after the mark can be investigated. Several paleontological studies have shown that natural trace levels of manganese in calcified tissue can be detected *in situ*, using cathodoluminescence (CL) (Sommer 1972; Barbin *et al.* 1991 a; Mazzoleni *et al.* 1995). Barbin *et al.* (1991 b) and Barbin (1992) used CL microscopy to detect manganese bound within growth layers of *Pecten* sp. and *Nautilus* sp. We have recently shown that immersion of abalone, *H. rubra*, in sea water enriched with manganese can be used to mark the growing edge of the shell. Manganese appears to substitute for calcium in the $CaCO_3$ lattice. The marks can be detected using CL, and reveal the mineralog of the layers by colour (Hawkes *et al.* 1996). Under CL, layers emit either an orange-red or yellow-green colour depending on whether manganese is incorporated into calcite or aragonite, respectively, because electrons are excited to different energy states in the two minerals (Sommer 1972). In this paper, we present the results of experiments to determine the optimum concentrations of manganese and immersion times required for this chemical marker to be useful for incremental analysis work on blacklip abalone for age validation studies.

Materials and Methods

Blacklip abalone, *Haliotis rubra*, were collected from Rickets Point, Port Phillip Bay, Victoria, Australia, and transported to the recirculating sea water system at the Zoology Department of The

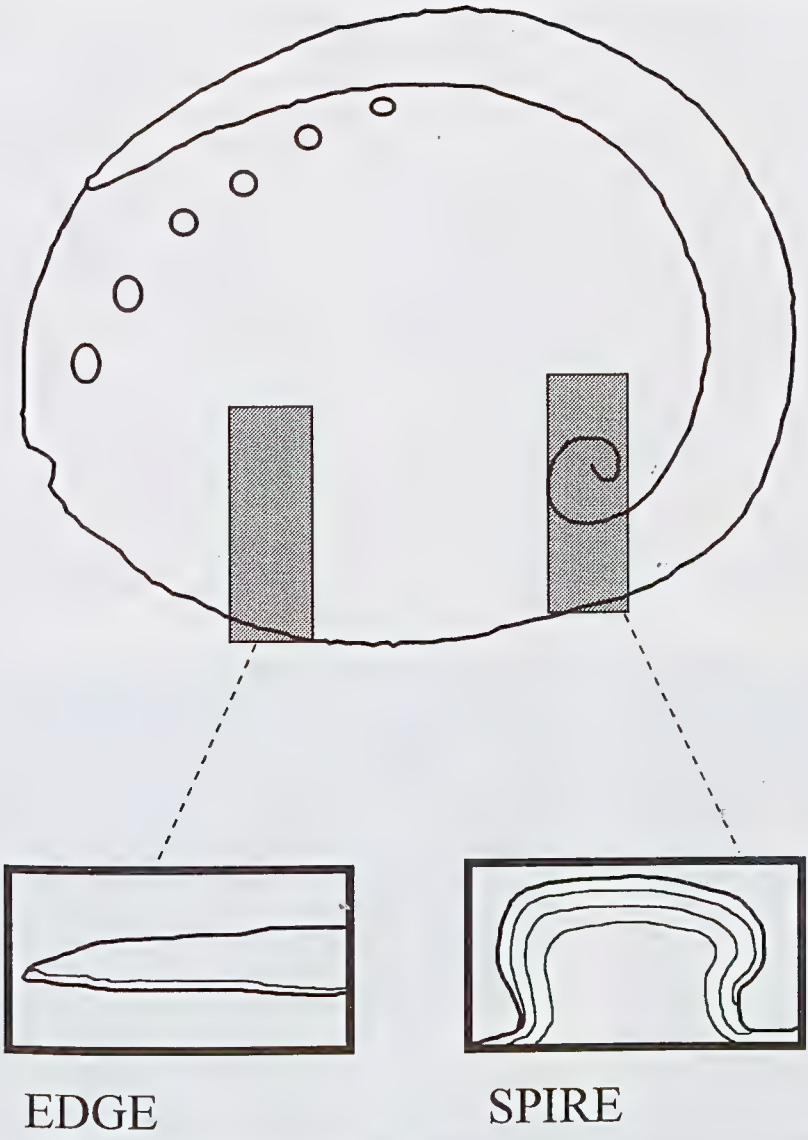

Figure 1. Ventral view of an abalone shell, showing where shell pieces were cut from the edge and spire to examine manganese marks.

University of Melbourne. All abalone were maintained within a temperature range of 17 ± 2 °C during the marking trials. An initial experiment was conducted to determine what concentrations of manganese were lethal to abalone immersed in manganese – sea water solutions, over a 48 h period. Abalone were placed in 20 L aerated buckets of sea water (5 per bucket) to which manganese ion was added (as $MnCl_2.4H_2O$). The amount of manganese added was varied with respect to Mn:Ca ratios, as it is known that increasing ratios of strontium to calcium affect the growth of molluscs (Buchardt and Fritz 1978). Concentrations of 189, 378, 945 and 1890 mg L^{-1} were used, corresponding to Mn:Ca ratios of 1:10, 1:5, 1:2 and 1:1. To observe any adverse effects of manganese treatments, abalone were rinsed and kept in normal sea water with regular feeding and flushing for a period of 10 days.

To determine the manganese concentrations and immersion times needed to produce useable time-marks for age validation, abalone were immersed for 48 or 96 h. All buckets contained 5 adult abalone ranging in length from 80 – 100 mm, with 2 or 3 juvenile abalone of size 50 – 70 mm. Based on the initial experiment, Mn:Ca ratios of 1:200, 1:100, 1:50, 1:20 and 1:10 (9.5 to 189.0 mg L^{-1} of $MnCl_2.4H_2O$) were used. To ensure that incorporation of manganese into the shell had been completed, the abalone were kept in normal sea water, with regular feeding and flushing for a "consolidation" period of 18 days. The abalone were frozen over night, thawed and the body separated from the shell. Pieces were cut from the spire and edge of the shell (Fig. 1), in areas corresponding to previous studies (Erasmus *et al.* 1994; Shepherd *et al.* 1995a, b; Day *et al.* 1995). The spire and edge pieces were embedded in polyester resin and the blocks were cut to reveal cross-sections of the shell. The surface was finely ground using three grades of emery paper and polished on a variable orbital polishing machine. The blocks were then cut to produce uniform 2-mm thick sections for analysis.

Manganese carbonate marks within the abalone shell sections were viewed under a beam of electrons in a vacuum, using optical microscopy, as described in Hawkes *et al.* (1996). Identifiable CL marks were scored for length and thickness (length scores ranged from 0 = no mark to 10 = very long; thickness scores from 1 = very fine to 5 = very broad). This system is based on a previous method to compare fluorochrome marks in shells (Day *et al.*, 1995). The total length included both orange-red (calcite) and yellow-green (aragonite) CL marks within a section (Hawkes *et al.* 1996). The thickness of marks was scored according to the width of the aragonite CL bands only. The

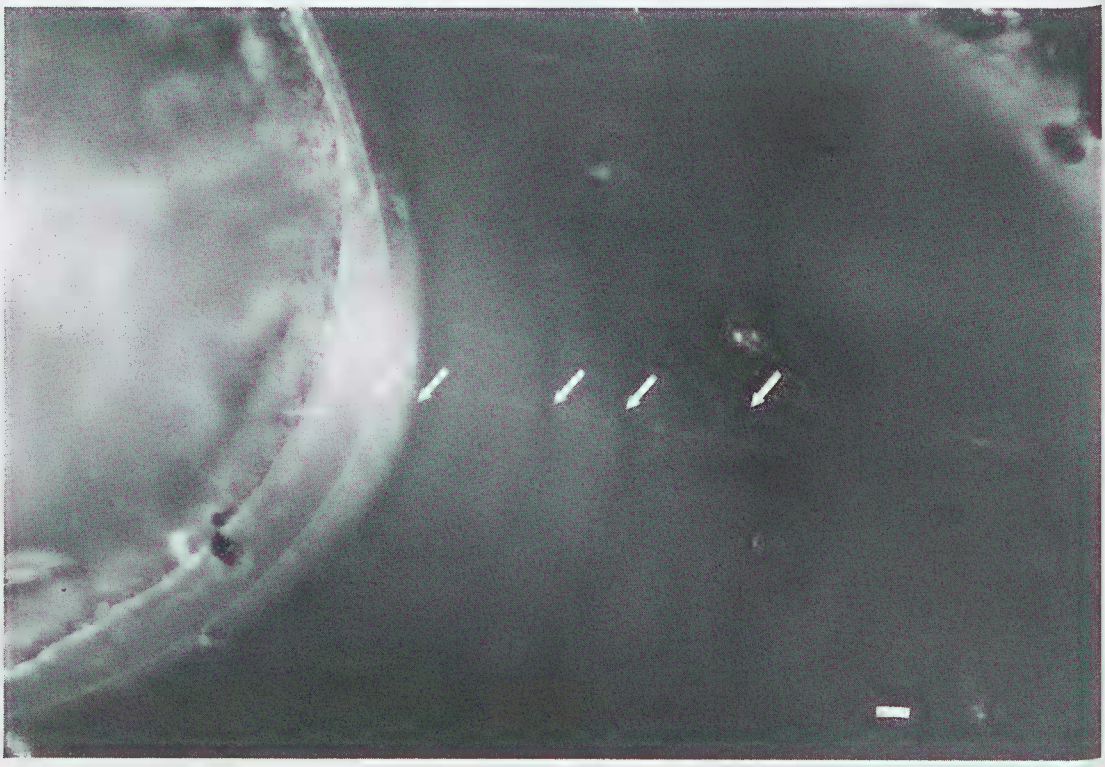

Figure 2. Cross-section of the spire of *Haliotis rubra*, photographed under normal reflected light and cathodoluminescence, showing natural spire layers (arrows), which have been used to age individual abalone, but require validation, inside a fine manganese mark (arrow-asterisk) formed when immersed in 189 mg L^{-1} of $MnCl_2.4H_20$ in sea water for 96 h. Scale bar = 50 mm.

average length and thickness of manganese marks were compared between treatments for both the spire and edge regions. As initial observations showed length and width scores were closely correlated, these scores were incorporated into a total score, where: total score = total length + width/4, in later trials. The scores were subjected to an analysis of covariance, with immersion time as a fixed factor, and manganese concentration as a covariant.

Using abalone collected from the same site, two further experiments were conducted in the same way to investigate the consistency of marking between experiments and to compare marking effectiveness at higher concentrations and longer immersion times (136 – 272 mg L^{-1} at 48 – 144 h).

To show that the marking technique works in the field an experiment was conducted on abalone collected at a depth of 3 m off Rickett's Point, Port Phillip Bay. One hundred and fifteen abalone were collected and stained at a concentration of 200mg L^{-1} over a 48 hr period. After the staining period abalone were kept in the holding pens on the reef for 14 days before they were collected and frozen to analyse the shells. In addition, 10 abalone shells that had not been stained were sectioned to check for natural fluorescent marks under CL.

Results

All abalone immersed in concentrations ≥ 945 mg L^{-1} of $MnCl_2.4H_20$ died within four days after the 48 hour immersion. Abalone at 379 mg L^{-1} appeared weak and stressed, with their body and shell raised off the bucket wall, but recovered quickly once flushed with normal sea water. The extension of tentacles beyond the shell rim, a rapid response to touch and a normal feeding rate indicated the

Table 1. ANOVA analyses of the effects of concentration and immersion time on scores of the length and width of manganese marks observed in edge and spire sections of abalone.

	Degrees of freedom	Mean square	F
Spire Mn length			
Concentration	1	4.929	1.303
Immersion time	1	10.596	2.801
Interaction	1	6.210	1.641
Error	66	3.783	
Spire Mn width			
Concentration	1	0.888	0.690
Immersion time	1	2.329	1.811
Interaction	1	0.000	0.000
Error	66	1.286	
Edge Mn length			
Concentration	1	96.574	16.450*
Immersion time	1	0.782	0.133
Interaction	1	2.478	0.422
Error	62	5.871	
Edge Mn width			
Concentration	1	36.740	25.161*
Immersion time	1	0.169	0.116
Interaction	1	3.577	2.449
Error	63	1.460	
Shell region			
Mn length	1, 138	124.570	24.409*
Mn width	1, 133	15.566	10.372*

abalone immersed at 189 mg L^{-1} of manganese were not stressed. In the latter two concentrations all abalone were alive when the experiment was concluded after 10 days. As it is difficult to measure any other sub-lethal effects, and we wished to ensure that long term deleterious effects were unlikely, manganese concentrations $\leq$ 189 mg L^{-1} were chosen to investigate the effects of varying concentration and length of immersion on the marks produced.

A cross-section of the spire region of a shell, double exposed under visible light and a beam of electrons is shown in Fig. 2. The yellow-green mark of manganese in aragonite is clearly visible within the section. By adjusting the intensity and amount of reflected light and CL, both normal growth layers and manganese induced marks may be observed simultaneously as shown, making this technique suitable for growth increment analysis.

Manganese induced marks within sections of the edge and spire of abalone shells varied in length, thickness and brightness. The mean length and thickness of marks in abalone immersed in 48 and 96 h treatments were plotted against the natural log of the concentrations of manganese in sea water (Fig. 3). Results for the length and thickness of scores were very similar. At the growing edge, the mean length and thickness of marks increased significantly with the concentration of manganese used (Table 1, Fig. 3a, b). Under the spire the maximum mean length and thickness of marks were produced at 38 and 95 mg L^{-1} in both the 48 and 96 h treatments, but no significant effect of concentration could be demonstrated (Table 1, Fig. 3c, d). In either shell region, no significant effect of immersion time on CL scores could be demonstrated (Table 1). CL marks were significantly longer and thicker at the edge than under the spire (Table 1).

To investigate whether marking ability varied between experiments, as found for fluorochromes (Day *et al.* 1995), a new batch of abalone was treated in a concentration of 189 mg L^{-1} for 48 h, as in the previous experiment. A comparison of the length of the marks between these experiments, which were carried out in winter and autumn, shows that edge length scores were higher than spire scores in the experiment conducted in winter, whereas similar scores, between shell regions, were obtained in the autumn trial (Fig. 4). The length of marks at the edge in the initial experiment were significantly ($p = 0.047$) higher than the latter, and vice versa ($p = 0.024$) for marks under the spire.

To investigate manganese marks in abalone subjected to higher concentrations and longer immersion times, abalone were treated to concentrations of 136, 204 and 272 mg L^{-1} of manganese in sea water baths for 48, 96 and 144 hours (2, 4 and 6 days). As the earlier results for length and width were similar, total scores were used. No animals died during the experiment. The variability of the total scores within each treatment is evident in the spread of the box plots in both shell regions (Fig.

Table 2. ANOVA analyses of the effects of concentration and immersion time on total scores of manganese marks observed in the edge and spire sections of abalone.

	Degrees of freedom	Mean square	F
Edge CL total score			
Concentration	2	11.201	1.322
Immersion time	2	243.784	5.169*
Interaction	4	33.394	3.942*
Bucket effect	1	7.803	0.921
Error	80	8.471	
Spire CL total score			
Concentration	2	3.586	0.518
Immersion time	2	0.709	0.102
Interaction	4	10.814	1.562
Bucket effect	1	0.056	0.008
Error	80	6.921	

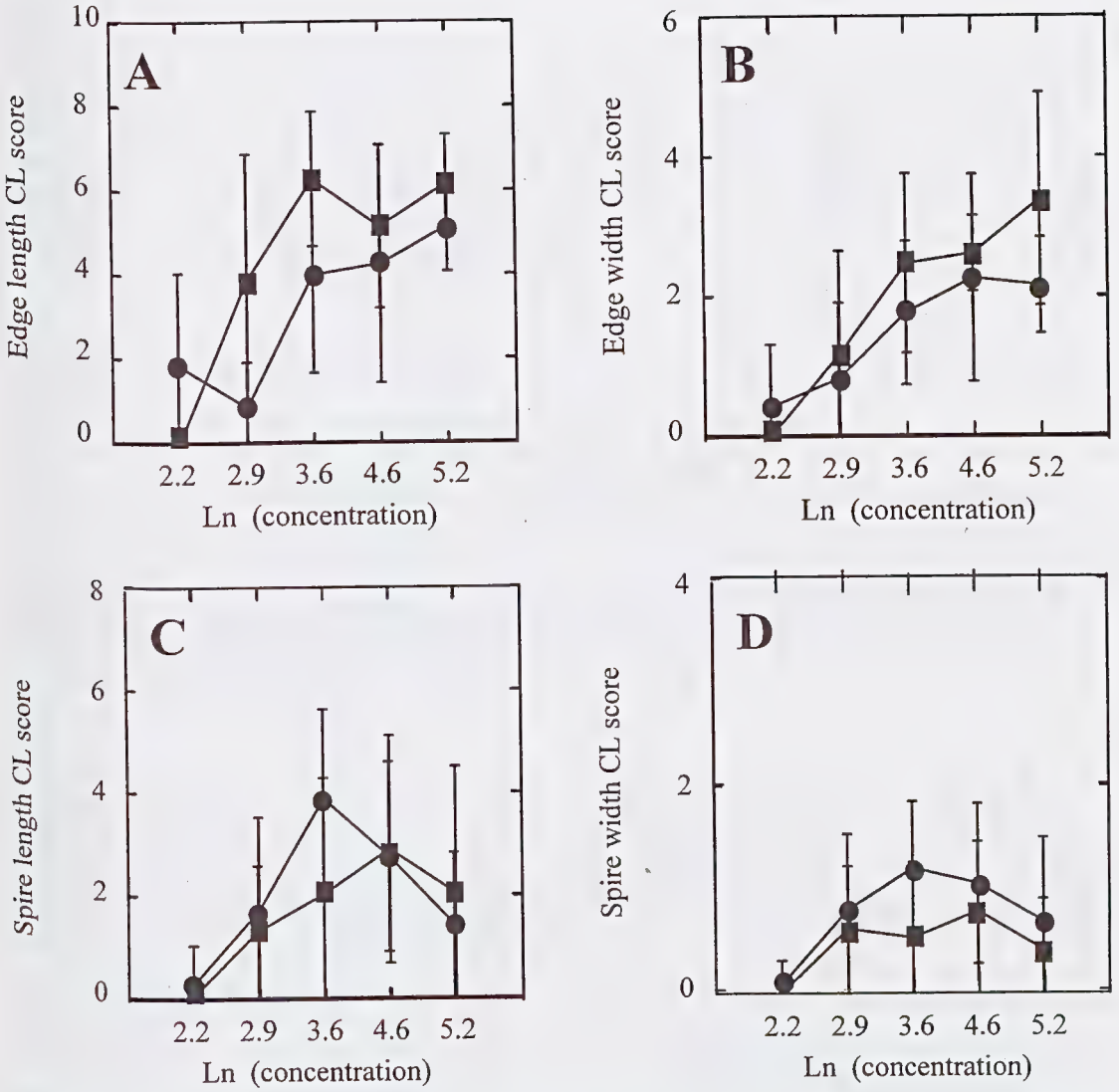

Figure 3. Comparison of scores for mean length (A) and mean width (B) at the edge and mean length (C) and mean width (D) at the spire of manganese marks in shells of abalone immersed in a range of concentrations of 9.5 to 189 mg L^{-1} (natural log) of $MnCL_2.4H_20$ in sea water for 48 h (●) and 96 h (■). Standard error bars are shown.

5). At the edge there was a significant interaction between immersion time and concentration (Table 2). This reflects the fact that scores at the growing edge increased with increasing immersion time, except in the most concentrated treatment (Fig. 5a). This may indicate some toxicity of manganese at this concentration over longer periods. The scores obtained for the spire were lower than the corresponding edge scores. They tended to decrease with concentration, and there was no obvious trend with immersion time (Fig. 5b). However, the highest mean score of 4.71 ± 3.37 was found in the middle range concentration (204 mgL^{-1}) with 144 h immersion.

In the field experiment, 108 abalone of the 115 marked were alive 14 days after the experiment. Clearly visible manganese marks were found in 90 sections of the edge region and 64 sections of the

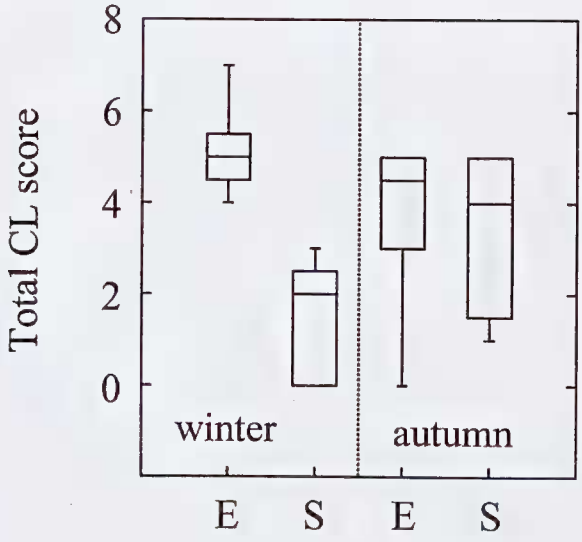

Figure 4. Boxplots of scores of manganese marks from the edge and spire shell region of abalone immersed in 189 mg L^{-1} of $MnCL_2.4H_20$ in sea water for 48 h during experiments conducted in late winter, 1994 and autumn, 1995. Sample size = 5 abalone for each treatment. The median, upper and lower quartile and range of the data are shown by each plot.

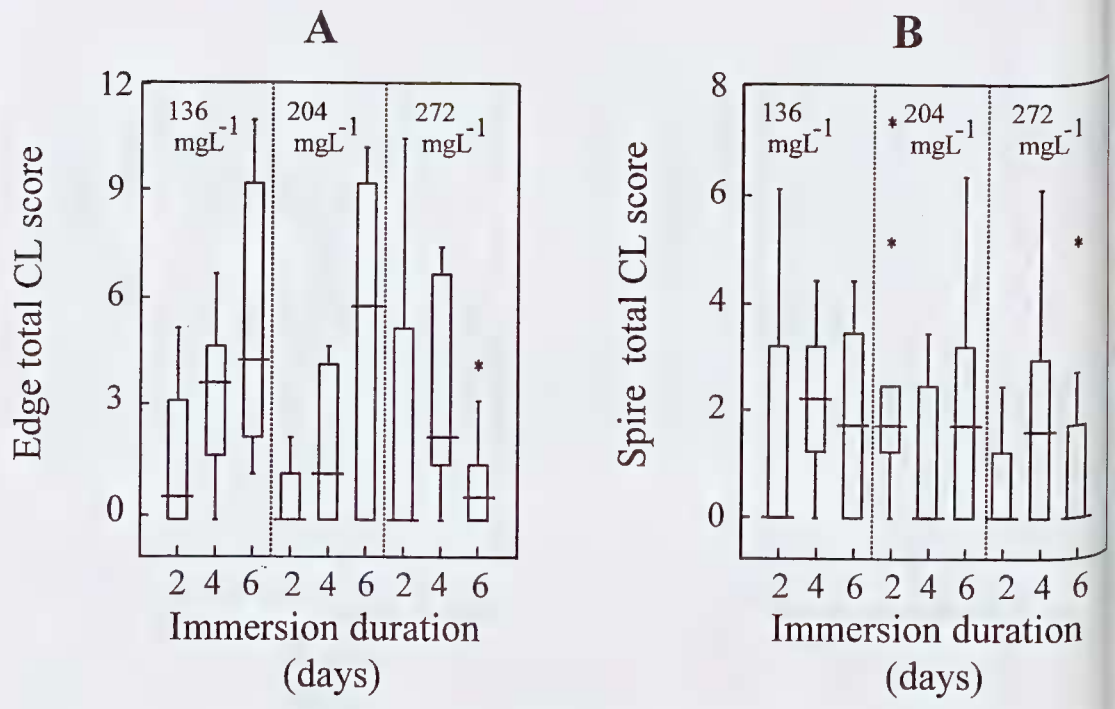

Figure 5. Boxplots of scores of manganese marks from the edge (A) and spire (B) shell region of abalone immersed in 136, 204 and 272 mg L^{-1} of $MnCL_2.4H_20$ in sea water for 48, 96 and 144 h (2, 4 and 6 d). Sample size = 10 abalone for each treatment. The plots are as in Figure 4, but here outliers are presented and denoted by stars.

spire region of these shells.

We observed natural luminescence from calcite and aragonite shell within unstained abalone specimens. In the prismatic layer both orange-red and yellow-green irregular banding was found. The marks observed in the nacre of adult abalone (80 – 100 mm) were mostly hair-like and irregular. However, numerous fine luminescent bands, or rings, were commonly found within the spire of juvenile abalone (30 – 50 mm), and occasional bands were recorded within the edge nacre. This natural CL banding was mostly dull and could be distinguished from the brightly marked layers produced in our staining experiments. However, the colours of natural CL bands where very similar to those of stained layers.

Discussion

Critical levels of manganese

For a marker to be useful it must not induce mortality (Behrens and Mulligan 1990). Manganese appears to be non-lethal except at doses much higher than those required to produce CL marks in shells. Our results suggest manganese chloride concentrations of less than 272 mg L^{-1} should be used to time-mark shells of blacklip abalone. We have obtained over 90% survival in all immersion experiments of blacklip abalone exposed to levels of manganese at or below 272 mg L^{-1}, and the marking results do not suggest any toxic effects except perhaps at 272 mg L^{-1} for 6 days. Pirker and Schiel (1993) found the survival rate of the abalone, *Haliotis iris*, marked with fluorochromes decreased when experiments were conducted in the field. Our *in situ* immersion experiment using manganese, at a concentration of 189 mg L^{-1}, demonstrated the same high survival rate as in the laboratory experiments.

Comparison of manganese marks

Marked shell layers were scored to determine if measurable differences in marking ability using manganese could be detected in abalone exposed to different concentrations and immersion times. In previous abalone marking experiments using fluorochromes Pirker and Schiel (1993) found brighter marks in juvenile abalone immersed over longer time intervals, and Day *et al.* (1995) found the effectiveness of marking at the shell edge increased with time and concentration. However, this was dependent on the fluorochrome used, the season and the batches of abalone used in experiments. Our experiments show no significant difference of CL mark scores between 48 and 96 h immersion in either shell area. There was a trend towards better manganese marks at the shell edge with increasing concentration, but scores were lower at the highest concentrations. Under the spire, concentration had less effect, and marking may reach a maximum at intermediate concentrations.

Calcite and aragonite polymorphs differ in the ease with which cations can be substituted within the crystal matrix (Sommer 1972; Tucker and Wright 1990; Carriker *et al.* 1991), although mineralogical control of trace element composition within mollusc shell appears to vary between species and ontogeny (Carriker *et al.* 1991; Mann 1992). In our study, manganese was only deposited within calcite layers in a short band at the growth margin in the edge sections, so that the length scores depended mostly on nacre deposition behind the growth margin, and width was scored across bands only within nacre. Thus the mineralogical control of manganese deposition would have had little or no effect on overall scores.

Substitution of Ca^{2-} with other elements is subject to physiological control (Mann 1992). Wilbur and Saleuddin (1983) concluded that the rate of ion movement across the mantle epithelia of molluscs was dependent on concentration, but only up to a saturation point. This suggests the rate of substitution of Ca^{2-} with Mn^{2-} across the mantle epithelium at the shell edge may have reached a saturation point at the highest concentrations. At 272 mg L^{-1} the concentration of manganese may induce some physiological stress, leading to less shell calcification and thus less marking. The significantly lower marking scores under the spire are presumably due to differences in deposition

rate, at least in part. The rate of shell deposition appears to be much faster at the growing margin than in the central region in molluscan shells (Wilbur and Saleuddin 1983). Furthermore, the characteristic flattened conical morphology of the abalone shell is a result of rapid deposition of oblique layers extending the outer margin and gradual deposition of horizontal layers thickening the inner regions (de Jong 1990).

The fact that under the spire the highest mean scores were found for intermediate concentrations may indicate that saturation limits within the extrapallial space under the spire are reached more rapidly, as the process of calcification within the inner region is slower, or that transport of manganese via the haemocoel is slow. If higher concentrations induced stress we would have expected this to have affected marking at the edge in the same way. However, perhaps blood flow to the spire region was reduced by higher concentrations.

The rate of shell growth may well be an important factor in determining CL scores in our experiments. The amount of Mn^{2+} deposited will depend on the amount of shell produced over the marking period. The lack of demonstrated effect of immersion time may be an artifact of the staining process, as water flow is necessarily reduced during staining. As abalone activity and growth is markedly affected by water flow the shell deposition process may slow during the experiment, so little marked shell is deposited after 48 h.

The variation within treatments may be large because some abalone were laying down shell rapidly while others were not growing at all. Leighton (1974) and Momma (1980) found variable growth between individual juvenile abalone in the laboratory over short periods, and we have observed growth spurts in juvenile abalone (Day, unpublished data). Field studies on abalone have also shown that the growth of individuals is highly variable both within and between seasons (Shepherd and Hearn 1983; Day and Leorke 1986; Day and Fleming 1992). Abalone are opportunistic feeders which rely on an unpredictable supply of drift algae, and their growth pattern appears to be related to this feeding regime (Day and Fleming 1992). Day and Fleming (1992) showed that growth characteristics may be dependent on previous feeding history, and Day *et al.* (1995) found that better fluorochrome marking was achieved with pre-fed animals, as compared to starved abalone, over a 24 h immersion treatment. However, these studies of growth and marking concern only growth at the edge of the shell. It remains to be seen whether food supply will influence deposition under the spire and therefore the ability to mark the shell for age estimation. Our field trial suggests that with our current methods we can expect to produce clear marks in about 60% of abalone which should be sufficient for validation trials.

Natural luminescent marks

If manganese is to be used to time-mark shell layers it is important that the marked layers can be distinguished from any natural marks. Sommer (1972), Barbin *et al.* (1991 a; b) and Barbin (1992) found that ambient levels of manganese produce natural luminescent growth zones in calcite in bivalves of marine, estuarine and freshwater habitats. The most intense marks were found in freshwater species (Sommer 1972). Yellow-green luminescence within nacreous layers was found only in hyposaline species; shells from marine habitats were non-luminescent (Barbin *et al.* 1991b; Mazzoleni *et al.* 1995). However, Barbin (1992) detected medium to dull CL marks in aragonitic layers of *Nautilus pompilius*, and weak luminescence in *N. macromphalus*. We have detected natural CL bands, similar in colour to our staining marks in abalone, especially juveniles, although natural marks were much duller and narrower than our marks. If these represent manganese induced marks, then the usefulness of manganese as a marker will depend on the local ambient levels of manganese and on the rate of mineralisation of shell during any doping period, as staining marks need to be much brighter than any natural marks.

Manganese marks versus fluorochrome marking

Other hard tissue markers have hitherto successfully stained the outer margin or growing edge region of the abalone shell, but not the spire (Pirker and Schiel 1993; Day *et al.* 1995). Age cannot

be validated by growth lines occurring at the outer margin of the abalone shell as these growth checks are irregular and have been related to disturbance during capture or handling (Day and Fleming 1992). In contrast, manganese is incorporated within both the spire and edge regions. In addition, the length of CL bands at the outer edge were 3 – 4 times greater in magnitude than most fluorochrome bands.

This difference may be related to the movement of the stains across membranes. Fluorochromes are large organic molecules that chelate to $CaCO_3$ crystals, while manganese is incorporated within shell layers by the substitution of Mn^{2-} for Ca^{2-} ion in the formation of carbonate crystals (Sommer 1972). It is expected that the Mn^{2-} ion would be easily transported to sites of calcification since the stoichiometry of these two anions is similar. However, the larger fluorochrome molecules may not move across various membranes as readily, and this may restrict their ability to stain inner areas of calcification. It is possible that fluorochrome and manganese staining of the edge region is caused by the direct absorption of the dye from sea water across the mantle epithelium into the extrapallial fluid as has been postulated for the deposition of calcium and trace elements deposition (Wilbur and Saleuddin 1983; Carriker *et al.* 1991). The formation of luminescent layers under the spire, however, indicates manganese is absorbed across the body wall into the haemocoele from where it is transported to the mantle epithelium throughout the body. In addition, the synthesis of aragonite in gastropods involves crystal formation below many layers of protein sheets (Nakahara 1981) and the larger fluorochrome molecules may not penetrate through these layers as easily as the Mn^{2-} ion.

The fact that manganese is incorporated into layers deposited under the spire, and that it can be detected *in situ* using CL, such that manganese marks and natural growth lines can be seen at the same time, makes this technique useful for age validation studies. Since simultaneous staining of the edge and spire regions occurs, this technique may also be useful in comparing the timing and frequency of shell layers between these areas.

Acknowledgments

We thank Rickard Officer, Warwick Nash, and Dan Morse for discussions. This study is part of a MSc study at The University of Melbourne and supported by a grant to R. W. D. from the Fishing Industry Research and Development Corporation. Experiments were conducted at the Zoology Department, The University of Melbourne, Queenscliff Marine Laboratories of the Marine And Freshwater Research Institute of Victoria and the Lara laboratories of Cheetham Salt Limited, and analysed at the School of Earth Sciences, The University of Melbourne. This paper is dedicated to the memory of Miss Mary Royle who translated the French papers cited in the text.

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